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Retrovirus insertion into herpesvirus *in vitro* and *in vivo*

(reticuloendotheliosis virus/Marek disease virus/long terminal repeat/T lymphoma)

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ABSTRACT **Retroviruses and herpesviruses are naturally occurring pathogens of humans and animals. Coinfection of the same host with both these viruses is common. We report here that a retrovirus can integrate directly into a herpesvirus genome. Specifically, we demonstrate insertion of a nonacute retrovirus, reticuloendotheliosis virus (REV), into a herpesvirus, Marek disease virus (MDV). Both viruses are capable of inducing T lymphomas in chickens and often coexist in the same animal. REV DNA integration into MDV occurred in a recently attenuated strain of MDV and in a short-term coinfection experiment *in vitro*. We also provide suggestive evidence that REV has inserted into pathogenic strains of MDV in the past. Sequences homologous to the REV long terminal repeat are found in oncogenic MDV but not in nononcogenic strains. These results raise the possibility that retroviral information may be transmitted by herpesvirus and that herpesvirus expression can be modulated by retroviral elements. In addition, retrovirus may provide a useful tool to characterize herpesviral function by insertional mutagenesis.**

Several interactions and synergisms between retroviruses and herpesviruses have been reported. Recently, it was shown that Marek disease virus (MDV), a chicken herpesvirus, can augment lymphoid leukosis induced by avian leukosis virus (ALV) (1). Infection of duck embryo fibroblasts (DEFs) with MDV has also been shown to transactivate the Rous sarcoma virus long terminal repeat (LTR) (2). The expression and replication of human immunodeficiency virus (HIV) can be accelerated by herpes simplex virus and human herpesvirus 6 (3–8). Furthermore, coinfection of cells by HIV and cytomegalovirus resulted in the expanded tropism of HIV (9).

In chickens, both retroviruses and herpesviruses are associated with naturally occurring neoplastic diseases. Nonacute retroviruses, represented by ALV and reticuloendotheliosis virus (REV), induce a variety of cancers in chickens after relatively long latency (10). Most frequently observed are bursal lymphomas; other diseases such as T lymphoma and erythroblastosis are also induced. In most cases examined, retroviral insertional activation of protooncogenes correlates with the development of tumors (11). Particularly relevant to this work is the T lymphoma, which involves c-*myc* activation by proviral insertion and is only induced by REV and not ALV (12, 13).

Herpesvirus-induced cancer in chickens is a frequent result of infection with MDV (14–16). MDV causes aggressive lymphomas of T-cell origin in various sites and enlargement of peripheral nerves due to infiltration of inflammatory or neoplastic lymphoid cells and is the only cancer for which a successful vaccine has been developed (17–20). MDV has a genome of 180 kilobases with two unique regions (U_L and U_S; Fig. 1*B*) flanked by inverted repeats (TR_L, IR_L, IR_S, and TR_S). There are three serotypes of MDV; type I (e.g., strains JM, MD, and GA) is oncogenic, whereas the vaccine strains types II (SB-1) and III (HVT) are not (19, 20). The oncogenic mechanism of MDV is not well understood, but propagation of type I MDV *in vitro* results in attenuation of its tumorigenicity. This process appears to correlate with a heterogenous expansion of TR_L and IR_L region (24–26). This expansion is principally due to the amplification of a 132-base-pair (bp) repeat element within the larger repeats (shown as vertical bars in Fig. 1*B*). It has been postulated that this amplification disrupts or downregulates a key viral gene involved in oncogenesis (27).

Although they differ in induction time and activation mechanism, the T-cell lymphomas induced by MDV and REV show strikingly similar tumor distributions (12, 13). It has also been shown that the REV- and MDV-induced tumor cells share common tumor-specific antigens, although whether these antigens are viral- or cell-encoded remains to be determined (28). We were therefore interested in interactions between these two viruses. In this communication, we will provide three lines of evidence demonstrating direct insertion of REV DNA into MDV genome. To our knowledge, this is the first report of retroviral insertion into the genome of a herpesvirus.

MATERIALS AND METHODS

Viruses, Cells, and Plasmids. The JM strain of MDV is the primary source of viruses used in this study (29). The preparation, propagation, cloning, and derivation of the attenuated JM viruses are as described in Witter and Offenbecker (30). Duck and chicken embryo fibroblasts were used for MDV infections by different strains of MDV. The *Bam*HI library of MDV was derived from the GA strain of MDV (21) and is a generous gift of M. Nonoyama (Tampa Bay Research Institute, St. Petersburg, FL). The REV LTR probe was derived from the *Sac* I–*Bam*HI fragment of the LTR and prepared as described (12).

Southern Hybridizations and MDV Genomic Library Construction. The Southern blot procedure and the construction of MDV genomic library in EMBL-3 λ vector are as described (12). The JM-Hi3 and -5 λ clones were isolated from the genomic library of passage-211 MDV/REV-coinfected material by hybridization with a REV LTR probe labeled with [^{32}P]dCTP (NEN) by nick translation (Amersham). High-stringency hybridization conditions were 42°C with 50% (vol/vol) formamide/5× Denhardt's solution/5× SSPE/0.1% SDS/denatured salmon sperm DNA (100 μg/ml). (1× SSPE = 0.18 M NaCl/10 mM sodium phosphate, pH 7.4/1

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Abbreviations: REV, reticuloendotheliosis virus; MDV, Marek disease virus; LTR, long terminal repeat; ALV, avian leukosis virus; HIV, human immunodeficiency virus; PFGE, pulsed-field gel electrophoresis; DEF, duck embryo fibroblast.

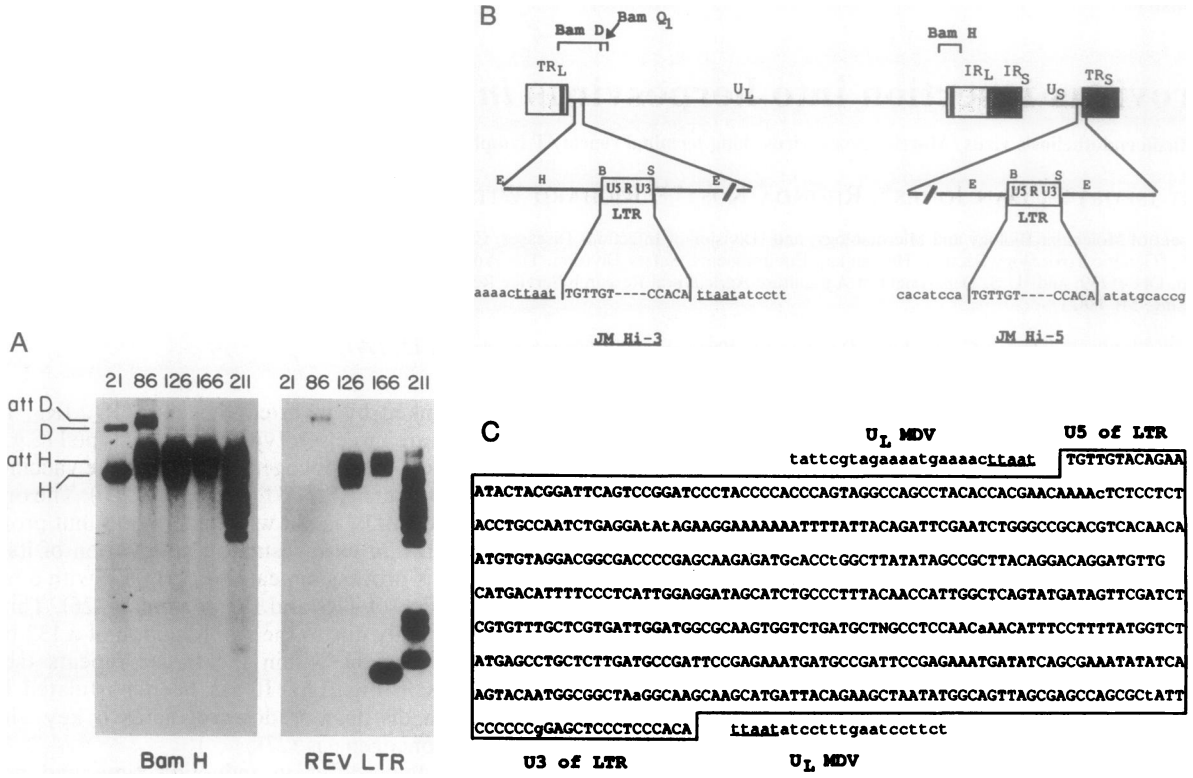

FIG. 1. REV insertions during the attenuation of JM MDV. (*A*) Southern blot analysis of passage-21, -86, -126, -166, and -211 JM strain of MDV. DNA was extracted from cells infected with MDV at different passages, digested with *Bam*HI, and Southern blot-hybridized with either nick-translated MDV *Bam* H fragment or an REV LTR-specific probe (*Sac* I–*Bam*HI fragment), as described (7). D and H refer to the location of wild-type (nonmutated) *Bam* D and H fragments, whereas attD and attH refer to the location of *in vitro*-passaged attenuated forms of *Bam*HI fragments D and H. (*B*) MDV genome and the REV insertions site in JM-Hi MDV. The MDV genome structure map is derived from Fukuchi *et al.* (21). *Bam* D, Q_1, and H refer to the locations in the genome where these *Bam*HI fragments map. The vertical bars in the TR_L and IR_L regions of the genome indicate the 132-bp repeat that is amplified during serial *in vitro* passage (10). The underlined sequence corresponds to the MDV sequence duplicated in JM-Hi3. The JM-Hi3 and -5 λ clones were isolated from an EMBL-3 genomic library of passage-211 MDV/REV coinfected material (lane 211 in *A*). Restriction enzyme sites are as follows: B, *Bam*HI; E, *Eco*RI; S, *Sac* I; H, *Hin*dIII. (*C*) DNA sequence of the LTR present in clone JM-Hi3. The inserted REV LTR is boxed. The uppercase letters represent perfect matches between REV LTR and the inserted sequences in the MDV genome and lowercase letters denote mismatches. REV LTR and MDV *Bam* D/H sequences are as described (refs. 22 and 23 and our data).

mM EDTA.) After hybridization, the blots were washed twice at room temperature in 2× standard saline citrate (SSC)/0.1% SDS. They were further washed twice at 68°C in 0.1× SSC/0.1% SDS. Low-stringency hybridization conditions were in the above hybridization solution at 37°C followed by two washes at room temperature with 2× SSC/0.1% SDS and two washes at 50°C with 1× SSC/0.1% SDS.

Inverse PCR and DNA Sequencing. The inverse PCR generally followed the method of Triglia and coworkers (31, 32). MDV genomic DNA was first digested with *Eco*RI (which does not cut in REV DNA) and then was ligated together in a large volume to create circular MDV DNA. The PCR was performed using primers homologous to the 5′ and 3′ end of REV LTR such that extension would proceed outward into the flanking MDV sequence on each side. Amplified products were subcloned using restriction sites in the LTR primers and sequenced. LTR primers were 19-mers incorporating the *Eco*RV (5′-CGCTGATATCATTTCTCGG-3′) or the *Bam*HI site (5′-GGGTGGGGTAGGGATCCGG-3′) of the REV LTR. Amplification was carried out in 100 μl containing 25 mM KCl, 20 mM Tris·HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.05% Tween, and bovine serum albumin (100 μg/μl). The PCR was performed for 35 cycles with 30 sec of denaturation at 95°C, 30 sec of annealing at 50°C, and 2 min of extension at 72°C.

Pulsed-Field Gel Electrophoresis (PFGE). The electrophoresis conditions and preparation of the DNA plugs are as described (33, 34). The DNA was separated in a 1% agarose/0.5× TBE gel for 20 h at 200 V with a 50- to 90-sec switch gradient using a Bio-Rad CHEF-DR II system. (TBE is 90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3.)

RESULTS

Recent REV Insertion into JM-Hi MDV. Our first evidence of REV insertion into MDV came from the characterization of an attenuated type I MDV, JM-Hi (i.e., high passage of JM virus). JM-Hi was developed by serial passage of a low-passage oncogenic strain of MDV (JM-Lo) in DEFs to obtain MDV with attenuated oncogenicity (24–26, 30). The current stock of JM-Hi MDV is at passage 211 and has significantly attenuated oncogenicity. During these passages, REV antigens were detected after passage 40 in the DEF culture, indicating a possible contamination by REV. The JM-Hi MDV has since been biologically purified from replicating REV by end-point dilution; however, REV insertion had already occurred. To monitor the course of both attenuation and REV insertion, DNA from DEFs infected with various passages of JM MDV was isolated, digested with *Bam*HI, and blot-hybridized to either a MDV *Bam* H fragment or a REV LTR probe under stringent conditions. The *Bam* H fragment encompasses portion of the large repeat and cross-hybridizes with a *Bam* D fragment. As shown in Fig. 1*A Left*, the *Bam* D and H regions showed evidence of TR_L/IR_L genomic expansion beginning at passage 86 (indicated by the diffuse bands labeled att D and H) and undergoing more drastic rearrangement in later passages. Fig. 1*A Right* shows hy-

bridization to REV LTR under high stringency. At passage 21 before REV contamination, no LTR signal was evident. REV insertions were first detected by the appearance of a faint band at passage 86; the REV signal increases in intensity upon further passages and generally follows the rearrangement pattern of *Bam* D/H region. At late passages (passages 166 and 211), insertions outside the *Bam* D/H region are also evident. A λ library of the MDV DNA isolated from passage-211 JM-Hi stock was constructed and screened with REV LTR probe under high stringency. Five clones were isolated and two, λJM-Hi3 and λJM-Hi5, were characterized in detail. Based on restriction mapping, DNA sequencing, and hybridization of the λ inserts to MDV DNA and to REV LTR, we were able to conclusively demonstrate physical linkage between REV LTR sequences and the MDV genome. In λJM-Hi3, (Fig. 1*B*) a solo LTR was found to be integrated downstream of the junction between the TR_L and the U_L. The last two nucleotides of the LTR are lost and there is a 5-bp direct duplication of the MDV sequence (ttaat) surrounding the LTR. These two features are hallmarks of authentic retroviral integration by REV (35, 36). The insertion in λJM-Hi5 also involves a solo LTR and is located near the boundary of U_S and TR_S. This insertion is associated with a deletion of the MDV genome resulting in noncontiguous U_S and TR_S sequences flanking the insertion site. As a result, the 5-bp duplication of the host sequence has been lost in this clone. The entire LTR sequence of JM-Hi3 is presented in Fig. 1*C*. The sequences of the two inserted LTRs are virtually identical to each other and share 98% homology with that of the T strain of REV (22, 23).

REV Insertion into MDV *in Vitro*. To offer more compelling evidence for REV insertion into MDV genome, we conducted a short-term coinfection experiment in DEFs. REV (10^4 viruses per ml)- and MDV-infected DEFs were cocultivated in the presence of fresh DEFs. Every 5 days the cells were passaged and mixed with fresh DEFs (ratio 1:1) for a total of 14 passages. A fraction of the cells at different passages were saved for PFGE. Under the PFGE conditions employed here, the duck chromosome (together with some supercoiled or trapped MDV episomes) would stay at the origin, whereas the MDV open-form minichromosome would migrate as a distinct band in the gel (33). The unintegrated REV DNA molecules would be too small to be retained in the gel. The gel was then Southern blotted and hybridized with a *Bam* D fragment probe to identify the position of MDV minichromosomes (Fig. 2*A Lower*). This was followed by hybridization with a REV LTR probe to detect possible integration events (Fig. 2*A Upper*). JM-Hi-infected cells, used as a positive control, revealed an MDV band that also hybridizes to REV LTR. As another control, JM-Lo (low-passage JM virus)-infected cells and REV-infected cells were mixed together before lysis and loading onto the gel (lanes JM-Lo + REV). No REV sequences were detected in MDV band, indicating that free REV DNA is not "trapped" by MDV minichromosomes. When the same experiments were conducted with REV/MDV-coinfected cells, REV LTR sequences were detected at high levels in late passages. However, REV hybridization is seen as early as passage 5. Trapping of MDV episomes in the well varies among preparations and, as a result, the REV LTR signals cannot be used to quantify the extent of insertion in each passage. Nevertheless, insertion clearly can occur within 5 weeks of initial coinfection. To confirm the PFGE data, the MDV minichromosome band from passage 14 was isolated (33) and the LTR junction fragments were amplified by inverse PCR using primers homologous to the 5′ and 3′ end of the REV LTR. These fragments were subsequently cloned and sequenced. The sequence of one representative clone is shown in Fig. 2*B* and its insertion site is indicated in the MDV map. This clone carries a REV LTR that has lost the terminal 2 nucleotides

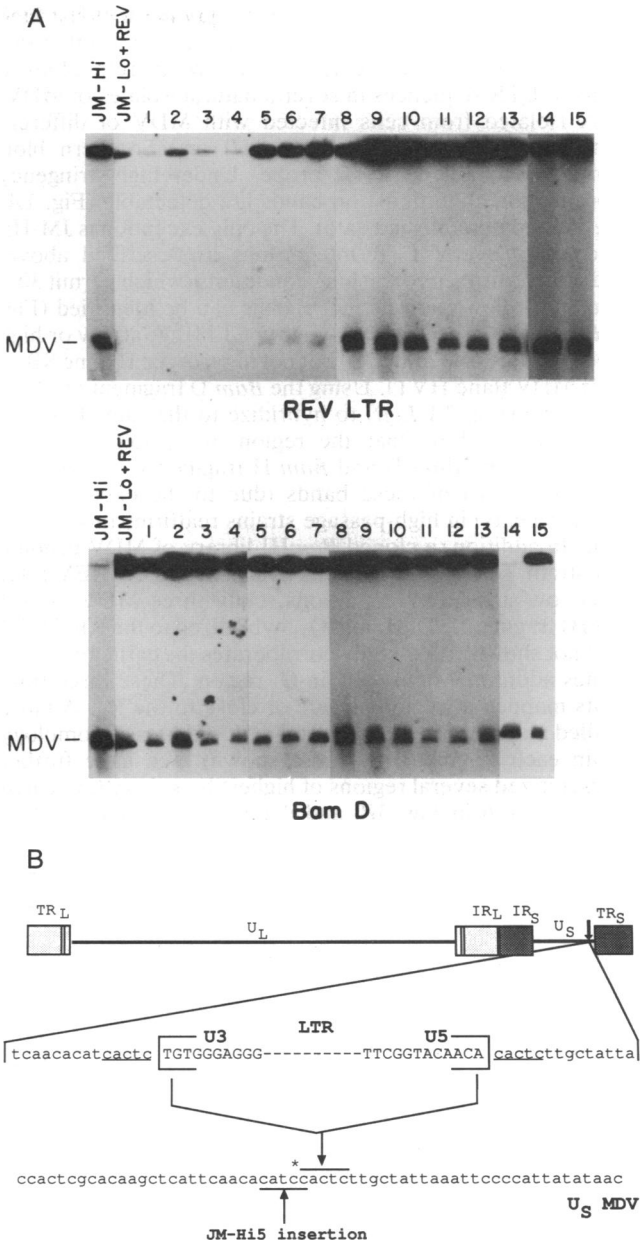

FIG. 2. REV insertions in *in vitro* cocultivation of REV and MDV. (*A*) PFGE analysis of REV/MDV coinfected cells was performed. After electrophoresis, the DNA in the gels was transferred to nitrocellulose filters by Southern blotting, and the filters were hybridized with either an REV LTR- or MDV *Bam* D-specific probe. JM-Hi refers to DNA from cells infected with the passage-211 virus as in Fig. 1*A*; JM-Lo + REV refers to DNA from JM passage-211-infected cells mixed with DNA from REV-infected cells prior to electrophoresis to serve as a control for nonspecific MDV/REV association. MDV refers to the migration position of the MDV genome. (*B*) The location and the junction sequence of a LTR insert in the cocultivation experiment. PFGE-purified MDV genomic DNA isolated from cells of the 14th passage after coinfection (see *A*) was amplified using the inverse PCR technique. This insertion maps to the same region of the U_S of MDV as found in JM-Hi above. The underlined letters are the duplicated MDV sequence and the sequence below represents the preintegration site. The asterisk indicates a cytosine nucleotide present in JM-Hi5 not found in the MDV DNA of passage-14 cells.

and is joined to duplicated MDV sequences. Remarkably, this LTR insertion occurs at a site immediately adjacent to the junction sequence found in the JM-Hi5 integration (Fig. 1*B*) but is in the opposite orientation.

REV Insertion into MDV *in Vivo*. Having demonstrated REV insertion into MDV *in vitro*, we wondered whether this type of interaction also occurred *in vivo*. We therefore looked for REV LTR sequences in several natural isolates of MDV. DNA isolated from cells infected with MDV of different serotypes was digested with *Bam*HI and Southern blot-hybridized to a REV LTR probe. Under high-stringency hybridization conditions, no bands are detectable (Fig. 1*A*, lane 21, and unpublished data). The only exception is JM-Hi, which has several LTR integrations as described above. Under low-stringency washing conditions (which permit 30% mismatch), however, distinct signals can be identified (Fig. 3*A Right*). They are seen only in type I MDV, at low or high passages (lanes JM and MD) but not in serotype II (lane SB-1) or III MDV (lane HVT). Using the *Bam* D fragment of MDV as a probe (Fig. 3*A Left*) to hybridize to the same blot, we could further show that the region of homology resides primarily in the *Bam* D and *Bam* H fragments. The heterogeneous pattern of these bands (due to the expansion of 132-bp repeats) in high-passage strains reaffirms this assignment. In addition, a cloned *Bam*HI library of MDV genome (GA strain, serotype I) (28) was hybridized to the REV LTR under low-stringency conditions. Only three MDV cloned *Bam*HI fragments, D, H, and Q_1, hybridized to the REV LTR (data not shown). This study corraborates the earlier data and defines additional homology in Q_1 region. These three fragments mapped at regions inside or close to the R_L. A more detailed mapping revealed several multiple sites of homology within each fragment (data not shown). We have further characterized several regions of highest homology (indicated by arrows 1–6 in Fig. 3*B*) and determined their respective sequences. We consider a stretch of >20 nucleotides with homology >70% to be significant. Fig. 3*B* illustrates the homology regions that we have identified thus far. They share 70–81% homology with the R and U3 regions of REV LTR. We are most persuaded by stretches 2–4, which are located within an 800-bp stretch of *Bam* D. Interestingly, stretch 1, which corresponds to the 3′ terminus of the 132-bp expansion unit, shares homology with the 3′ end of R region of the LTR. Most of these individual sequence stretches are calculated to occur randomly about once every 10^7–10^8 bases. Therefore, such a clustering of these sequences in particular regions of the MDV genome is unlikely to occur by chance. Since we have not determined the entire sequences of *Bam* D, H, and Q_1, there are likely other homology regions not reported here. These analyses provide suggestive evidence for REV insertions in the progenitor strains of serotype I MDV genome. Hybridization of MDV DNA with ALV LTR or murine leukemia virus LTR (data not shown) under similar conditions failed to detect any signal. Likewise, computer homology search of known MDV sequences with ALV, murine leukemia virus, and REV LTRs revealed significant homology over long stretches of sequence only with REV. This shared homology may reflect their long history of natural coexistence and their common T-cell tropism. Indeed, the chicken syncytial virus strain of REV was originally isolated from chickens with Marek disease (37).

DISCUSSION

In this report, we presented evidence of two cases in which REV integrated directly into MDV. We also suggest that

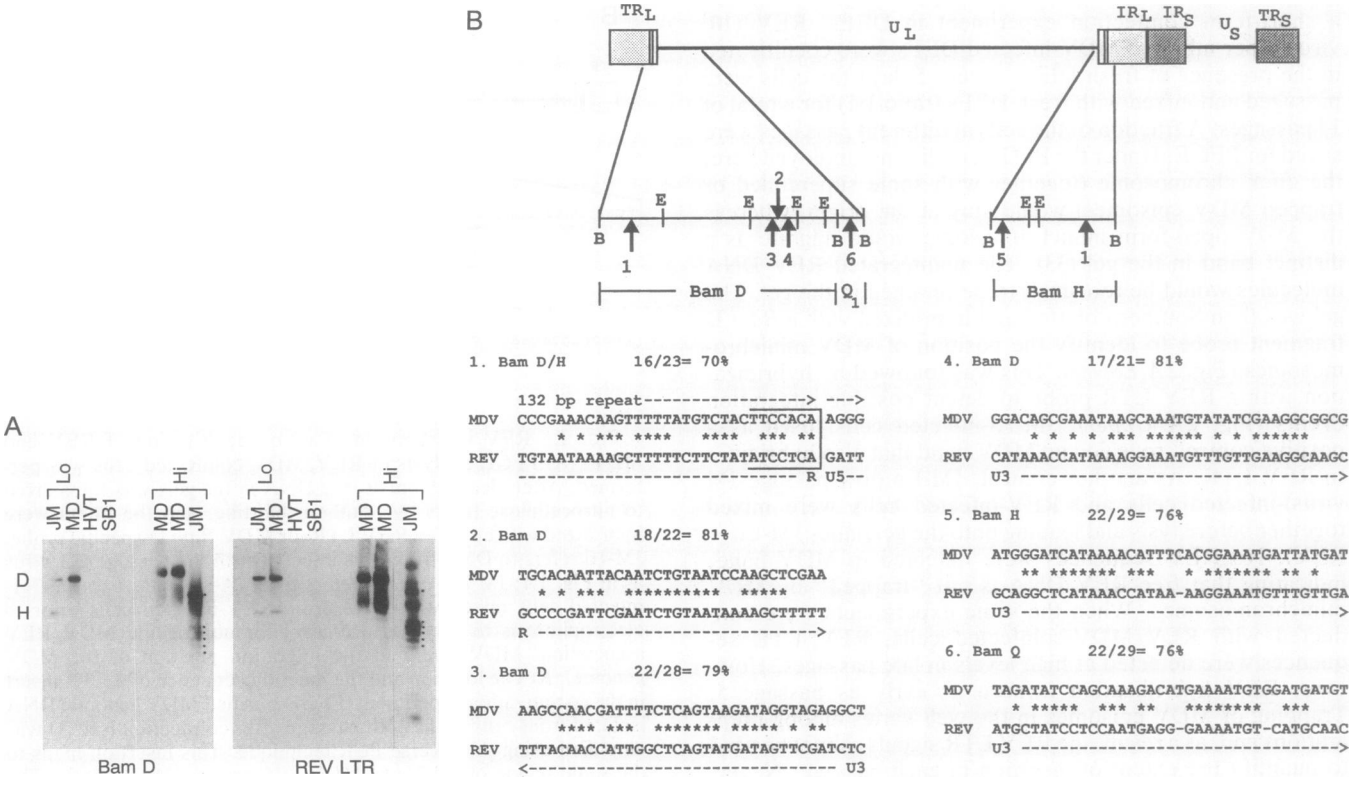

FIG. 3. REV-related sequences in wild-type MDV. (*A*) Hybridization of MDV DNA of various strains with MDV *Bam* D and REV LTR probes. DNA isolated from cells acutely infected with various strains of MDV was digested with *Bam*HI and analyzed by Southern blot hybridization. Hybridization with *Bam* D was conducted under high-stringency conditions, whereas hybridization with REV LTR was under low stringency. JM and MD (Lo) are two serotype I oncogenic strains of MDV at low passage; MD and JM (Hi) are their high-passage nononcogenic counterparts. Two isolates of MD-Hi were used. SB1 and HVT are natural isolates of nononcogenic MDV. D and H denote where *Bam* D and H fragments should migrate in the gel. The dots indicate bands of JM-Hi detected by both probes. (*B*) Sequence homology between REV LTR and wild-type MDV. Specific regions in *Bam*HI fragments D, H, and Q_1 that were homologous to REV LTR were isolated, subcloned, and sequenced. Sequenced regions were aligned with the corresponding region of the REV LTR using the IBI MacVector program. Also included in *B* is a map showing the locations in the *Bam* D, H, and Q_1 fragments of the specific regions of homology identified and sequenced.

REV homologous sequences in type I MDV represent retroviral insertions that occurred during the natural evolution of pathogenic serotypes. That these REV-related sequences are preserved in several viral strains and found in MDV isolated from different parts of the world may indicate a functional significance. The REV-like sequences are found principally in the R_L region and adjacent regions of the U_L. Since the inserted REV LTR contain elements that bind transcriptional factors and control tissue-specific transcription, it is tempting to speculate that some REV-related sequences may be involved in modulating the MDV expression in specific cell types. In this regard, it is interesting to note that REV and MDV share similar tissue tropism for oncogenesis and that the 3′ end of the 132-bp repeat, implicated in the control of oncogenicity, shares some homology with REV LTR.

We also provided evidence that REV inserted into the MDV genome during *in vitro* attenuation of JM MDV. These newly acquired sequences share 98% homology with REV LTR (22, 23). Whether REV insertion contributes to the attenuation process is not clear. It is, however, worth noting that passage-211 stock is dominated by viruses carrying REV insertions (30). This suggests that some of the insertions may confer an *in vitro* growth advantage to the virus. To this end, we have now clonally isolated the MDV carrying the JM-Hi5 insertion and found that it indeed has an enhanced growth rate when compared to the wild-type MDV.

Finally, we were able to recreate the insertions *in vitro* in cocultivation experiments. The insertions appear to be mediated by the retroviral integration machinery, followed by herpesvirus-induced homologous recombination (38), resulting in mainly solitary LTR insertions. LTR insertion may disrupt and inactivate a herpesvirus gene or may activate a herpesvirus gene through LTR promoter/enhancer elements. In either case, retrovirus can be exploited as an insertional mutagen to study herpesvirus gene function.

We have now extended this study to other retrovirus and herpesvirus system, in particular, REV insertion into HVT (a natural isolate of non-oncogenic MDV) and ALV insertion into MDV. In both cases, retroviral integration can be identified as early as the second passage after coinfection (R.I. and R.W., unpublished result). These results conclusively demonstrate the ability of retrovirus to insert into herpesvirus genome and further suggest that this phenomenon is not restricted to the REV/MDV system. We would predict integration in other systems where coinfection of the same target cell by both viral types takes place (e.g., HIV and human herpesvirus 6). In these systems, stable transmission of retroviral information by herpesvirus may have important clinical implications.

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